

Effect of Superoxide Dismutase on Infarct Size and Postischemic Recovery of Myocardial Contractility and Metabolism in Dogs

JOHAN VANHAECKE, MD, FRANS VAN DE WERF, MD, ALADAR RONASZEKI, MD, WILLEM FLAMENG, MD, EMMANUEL LESAFFRE, PhD, HILAIRE DE GEEST, MD

Leuven, Belgium

The effects of superoxide dismutase treatment on infarct size, postischemic recovery of contractile function and tissue content of high energy phosphates were examined in a canine model of myocardial ischemia and reperfusion. Ischemia was induced by thrombotic occlusion of a coronary artery and reperfusion was achieved by intravenous thrombolysis. Average duration of ischemia was 90 min. Fifty closed chest anesthetized dogs were randomized to receive either superoxide dismutase (34,000 IU/min intravenously) or placebo, starting approximately 30 min before and continuing for 30 min into the reperfusion phase.

Left ventricular ejection fraction and regional segmental shortening of the postischemic area were calculated from contrast angiograms after 4 h, 48 h and 1 week of reperfusion. Tissue content of high energy phosphates was determined from transmu-

ral biopsy after 4 h and 1 week. Infarct size was measured by planimetry of dye-stained heart slices.

In the superoxide dismutase and placebo-treated groups, respectively, the mortality rate was 25% and 16%, collateral flow 20 ± 10 and 23 ± 18 ml/min per 100 g, area at risk $25 \pm 6\%$ and $26 \pm 7\%$ of the left ventricle and infarct size $28 \pm 19\%$ and $36 \pm 27\%$ of the area at risk. Multiple regression analysis failed to show any beneficial effect of superoxide dismutase treatment on infarct size. Left ventricular ejection fraction, regional segmental shortening of the postischemic area and tissue content of high energy phosphates recovered to a similar extent and at a similar rate in both treated and placebo groups up to 1 week after reperfusion. Thus, in this model of coronary occlusion and reperfusion, superoxide dismutase treatment is of no benefit.

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Despite evidence of the generation of cytotoxic oxygen free radicals during myocardial ischemia and reperfusion, debate continues on the role of these radicals in myocardial stunning, reperfusion arrhythmias and irreversible reperfusion injury (1). Accordingly, there is no consensus on the effect of therapy with superoxide dismutase, a free radical scavenging enzyme, in the setting of coronary reperfusion.

The recovery of postischemic contractile function in dogs after 15 min of coronary occlusion is enhanced by pretreatment with superoxide dismutase plus catalase (2-4). It remains to be determined whether this benefit persists if the administration of free radical scavengers is delayed until the time of reperfusion. When the duration of ischemia is prolonged to 2 h to produce subendocardial infarction, superoxide dismutase given with catalase at the time of reperfusion does not alleviate the postischemic contractile dysfunction (5).

The issue of infarct size limitation by administration of superoxide dismutase remains unresolved as well, with some groups (6-11) consistently claiming beneficial effects and

others (5,12-16) reporting the lack thereof. Most of these discrepancies may be explained by differences in experimental design, although it is difficult to account for all the diversity in available data on this basis only (1).

The purpose of this study was to examine the effects of superoxide dismutase treatment in a coronary reperfusion setting mimicking the clinical situation. We used a canine model with 90 min of coronary occlusion, where reperfusion was achieved by thrombolysis and superoxide dismutase treatment was started simultaneously with the thrombolytic infusion. We specifically examined the effect of treatment on infarct size and postischemic recovery of contractile function. The latter was measured early (4 h) and late (48 h and 1 week) so that the effects on myocardial stunning and the results of infarct size limitation might be differentiated. We also measured, as a corollary to contractile function, the myocardial content of high energy phosphates after 4 h and 1 week of reperfusion.

Methods

Surgical preparation. Fifty mongrel dogs of either gender, weighing 14 to 27 kg, were premedicated with Hypnorm (10 mg fluanisone/0.2 mg fentanyl per ml), 0.25 ml/kg body weight intramuscularly, and anesthetized with sodium pentobarbital, 15 mg/kg intravenously. After endotracheal intubation, the lungs were ventilated with a 50/50 mixture of

From the Laboratories of Experimental Cardiology and Experimental Cardiac Surgery and the Division of Biostatistics, University of Leuven, Leuven, Belgium. This study was supported by a grant from Grunenthal GmbH, Stolberg, Germany.

Address for reprints: Johan Vanhaecke, MD, Department of Cardiology, University Hospital Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium.

oxygen and room air with use of a Bird Mark 7 respirator. A cannula was inserted into the left external jugular vein for administration of drugs and fluids and into the left femoral artery for measurement of blood pressure and withdrawal of blood samples; angiographic catheters were advanced through the left common carotid artery. Electrocardiographic (ECG) lead II was monitored on an oscilloscope.

Study Protocol

After baseline measurements of heart rate and blood pressure, contrast angiograms of the left ventricle and left coronary artery were obtained. Subsequently, a copper coil attached to a guide wire was advanced under fluoroscopic control through the carotid artery into the left anterior descending coronary artery. The formation of a thrombotic coronary occlusion within 5 to 10 min was confirmed by repeat angiography.

Forty-five minutes later, hemodynamic measurements were repeated and regional myocardial blood flow was determined by injection of radioactive microspheres. A second left ventricular angiogram was obtained and persistence of total coronary occlusion was verified by repeat coronary angiography. At this point, the dogs were randomized to receive either saline placebo or active treatment with superoxide dismutase (yeast-derived human recombinant copper-zinc superoxide dismutase, [Grunenthal]: specific activity 3,400 IU/mg) (17). The operators did not know the group assignment until data analysis was completed. The randomization protocol was such that the first 16 dogs would be equally divided between placebo and superoxide dismutase groups.

Thrombolysis. At 60 min after occlusion, thrombolytic infusion was started in all dogs. To this end, we used saruplase (recombinant unglycosylated full length human prourokinase [Grunenthal]; specific activity 164,000 U/mg) intravenously at a dose of 20 μ g/kg per min. The patency status of the left anterior descending coronary artery was checked angiographically every 5 to 10 min until a normal distal runoff of contrast dye was obtained; this occurred on average 30 min after the start of the thrombolytic therapy (see Results). The copper coil was then removed to prevent reocclusion after completion of the saruplase infusion, which was continued for a total of 60 min (that is, approximately 30 min into the reperfusion period).

Superoxide dismutase. Simultaneously with the thrombolytic infusion, either placebo or active treatment was started. Active treatment consisted of an intravenous infusion of superoxide dismutase at 10 mg/min (34,000 IU/min) as a 6 mg/ml solution; in the placebo group, the same volume of saline solution was given. The superoxide dismutase or placebo infusion was continued for 1 h; superoxide dismutase-treated dogs thus received a total dose of 600 mg (2.04 million IU), which was about equally divided between the last 30 min of ischemia and the first 30 min of reperfusion.

Hemodynamic measurements, ventricular and coronary angiography and myocardial biopsy. In the first 16 dogs, the duration of reperfusion was limited to 4 h. At that time, hemodynamic measurements as well as ventricular and coronary angiography were repeated. The chest was then opened through a left lateral thoracotomy, the heart exposed and transmural needle biopsy specimens (Tru-Cut biopsy needle, Travenol Laboratories) were obtained in duplicate from the center of the postischemic left anterior descending artery area and from control myocardium supplied by the left circumflex coronary artery. The heart was subsequently arrested with intravenous potassium chloride and excised for further processing. In this group of experiments, there were no premature deaths.

In the remaining 34 dogs, after reperfusion was established, skin wounds were sutured and the dogs were returned to their cage. Hemodynamic measurements as well as ventricular and coronary angiograms were repeated after 2 days and again after 1 week during a final experiment that also included taking myocardial biopsy specimens as just described.

Data Analysis

Hemodynamics. Heart rate was calculated from the electrocardiogram (ECG). Systolic and diastolic aortic blood pressure and left ventricular end-diastolic pressure were measured through a fluid-filled catheter and averaged over five consecutive beats.

Infarct size. After excision of the heart both coronary ostia were cannulated as was the left anterior descending coronary artery at the site of previous occlusion, which was identified from the angiograms. This artery was perfused with Ringer's solution; the ostia were perfused at the same pressure with a mixture of Ringer's solution and Evans blue. After 2 min the left anterior descending artery perfusing solution was changed to a triphenyltetrazolium chloride solution (18) at 37°C for 10 min. Finally, the heart was fixed by perfusing the left anterior descending artery area for another 5 min with 2% glutaraldehyde and both coronary ostia with a mixture of 2% glutaraldehyde and Evans blue.

After the right ventricle, the atria and valvular structures were removed, the isolated left ventricle was cut in 1 cm thick slices perpendicularly to the long axis. With use of calibrated color pictures of these slices, the left ventricular ring, the perfusion area of the left anterior descending coronary artery and the infarct area were reproduced with black ink on a transparent plastic sheet. Total left ventricular area, left anterior descending perfusion area and infarct area were calculated by automated planimetry (19), performed on the black ink reproductions of the original color pictures, with use of a Quantimet 900 image analyzer (Cambridge Instruments, Ltd.). The size of the left anterior descending perfusion area and of the infarct area were expressed as a percent of the total left ventricular area.

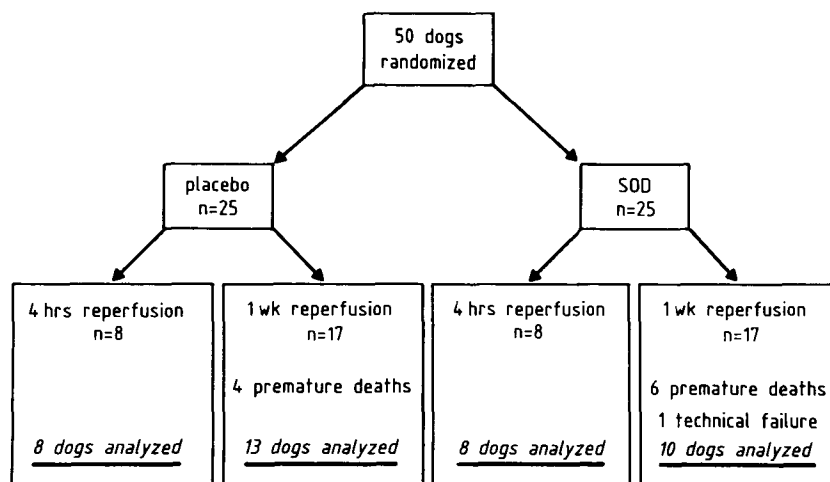


Figure 1. Experimental protocol. SOD = superoxide dismutase.

Collateral flow. Regional myocardial blood flow was measured with the tracer microsphere technique. We used microspheres with a 15 μ m diameter labeled with cerium-141, tin-113, ruthenium-103 or niobium-95 (NEN Chemicals GmbH). Approximately 5.10^6 microspheres per measurement were injected into the left ventricular cavity through an angiographic pigtail catheter, while a reference blood sample was withdrawn from the descending aorta. At the end of an experiment, the left anterior descending and circumflex artery regions of the left ventricular slices (as indicated by the differential dye staining) were separated and subdivided into subepicardial, mid-myocardial and subendocardial segments, which were cut into multiple tissue samples. Regional myocardial blood flow was then quantified by the method of Domenech et al. (20). Transmural blood flow (ml/min per 100 g) in the left anterior descending and circumflex artery regions was calculated as the weighted average of all the samples in the respective region.

Ventricular wall motion. Global and regional left ventricular wall motion were analyzed by computer processing of end-diastolic and end-systolic frames of the contrast angiogram (21). Global left ventricular ejection fraction was calculated and nine segments were identified for the study of segmental wall motion according to the method of Leighton et al. (22). As a measure of segmental wall motion in the left anterior descending artery area, we calculated the mean percent shortening of three hemiaxes that spanned the involved portion of the anterior wall.

High energy phosphate content. The biopsy specimens were immediately frozen in liquid nitrogen and stored at -80°C until further processing. After lyophilization, homogenization, extraction with 0.6 N perchloric acid and neutralization with potassium bicarbonate, the creatine phosphate and adenosine triphosphate (ATP) content were determined by using a bioluminescence assay (ATP bioluminescence CLS assay, Boehringer Pharma). For each measurement, the values from the two biopsy specimens were averaged.

Statistics. Differences between superoxide dismutase and placebo groups were evaluated by using multiple regression

analysis to correct for variables other than treatment allocation that may influence the outcome variable. When analyzing infarct size, we (23) previously demonstrated that only the size of the area at risk (as a percent of the left ventricle) and the amount of collateral flow (as a percent of flow in the normally perfused area) have a significant predictive value; these variables were included in the multiple regression analysis together with the duration of reperfusion (4 h or 1 week).

In evaluating postischemic global and regional contractility, we corrected for heart rate, systolic blood pressure, left ventricular end-diastolic pressure, infarct size (as a percent of the left ventricle and of the area at risk) and global and regional contractility in preischemic control conditions. When comparing myocardial high energy phosphate content, we corrected for infarct size (as a percent of the area at risk) and for the high energy phosphate content in normally perfused myocardium.

Calculation of the statistical power of the infarct size analysis was based on a univariate comparison between the two treatment groups using the Wilcoxon test for inference. Values are given as mean values \pm 1 SD. Nominal significance level was set at $p = 0.05$. For power calculations, we used the computer package Power Pack (24); all other calculations were done using the SAS statistical package (25).

Results

Mortality. Of the 50 dogs randomized, 10 died prematurely before completion of the protocol; 4 deaths were in the placebo group and 6 in the superoxide dismutase group. All of these premature deaths occurred in dogs that were meant to survive for 1 week. One additional experiment in the superoxide dismutase group could not be analyzed because of a technical failure with the dye perfusion staining. Thus, the final analysis involved 39 dogs (Fig. 1).

Table 1. Hemodynamic Data in 39 Dogs

	Control	Occlusion	Reperfusion		
			4 h	48 h	1 Week
Placebo (no.)	21	21	8	13	13
HR (beats/min)	84 ± 29	106 ± 22	141 ± 40	79 ± 18	87 ± 21
BP (mm Hg)	101 ± 19	107 ± 23	106 ± 21	89 ± 19	91 ± 21
LVEDP (mm Hg)	7 ± 2	10 ± 5	9 ± 5	10 ± 5	9 ± 4
SOD (no.)	18	18	8	10	10
HR (beats/min)	84 ± 24	109 ± 34	146 ± 30	84 ± 25	84 ± 24
BP (mm Hg)	101 ± 16	109 ± 19	97 ± 18	85 ± 16	96 ± 19
LVEDP (mm Hg)	7 ± 2	9 ± 3	7 ± 6	9 ± 4	9 ± 3

BP = systolic blood pressure; HR = heart rate; LVEDP = left ventricular end-diastolic pressure; SOD = superoxide dismutase.

Duration of ischemia. The duration of ischemia was 90 ± 11 min (range 76 to 119) in the placebo group and 90 ± 9 min (range 72 to 109) in the superoxide dismutase group.

Hemodynamics (Table 1). There were no differences between the placebo and superoxide dismutase groups in heart rate, systolic blood pressure or left ventricular end-diastolic pressure at any time during the experimental protocol. In both groups, heart rate had increased after 4 h of reperfusion. After 48 h, systolic blood pressure was somewhat lower. After 1 week of reperfusion, hemodynamic values were similar to those in the control period.

Size of the area at risk. The perfusion bed of the left anterior descending coronary artery beyond the site of occlusion constituted $25 \pm 6\%$ (range 14% to 37%) of the left ventricle in the superoxide dismutase group and $26 \pm 7\%$ (range 14% to 38%) in the placebo group.

Collateral flow. During occlusion, transmural collateral blood flow in the left anterior descending artery area averaged 20 ± 10 ml/min per 100 g (range 7 to 42) in the superoxide dismutase group and 23 ± 18 ml/min per 100 g (range 6 to 67) in the placebo group.

Infarct size. Infarct size expressed as a percent of the area at risk was $28 \pm 19\%$ (range 3% to 69%) in the superoxide dismutase group versus $36 \pm 27\%$ (range 3% to

85%) in the placebo group. The individual results for infarct size and transmural collateral flow are plotted in Figure 2.

With infarct size as the dependent variable, multiple regression analysis was performed, including the size of the area at risk, amount of collateral blood flow, treatment allocation and duration of reperfusion. As a result of this analysis, we were unable to demonstrate any group effects (that is, infarct size was not influenced by the duration of reperfusion [4 h or 1 week] or by treatment allocation [superoxide dismutase or placebo]). The only variables that were important in predicting the size of the infarct were the size of the area at risk and the level of collateral blood flow during ischemia.

On the basis of a univariate analysis comparing the two treatment groups and using the Wilcoxon test for inference (significance level = 0.05), we calculated a statistical power for this study of approximately 30%, 58% and 75% for infarct size reductions (from placebo to superoxide dismutase) of 30%, 50% and 60%, respectively. The power associated with multiple regression analysis is usually higher than these values, but is subject to more uncertainty because of the uncertainty with which the coefficients of the other risk factors are estimated.

Figure 2. Relation between infarct size expressed as a percent of the area at risk (I/P) and transmural collateral (COLL.) blood flow during left anterior descending coronary artery occlusion. Closed symbols indicate the active treatment group; open symbols indicate the placebo group; triangles indicate 4 h of reperfusion; circles indicate 1 week of reperfusion.

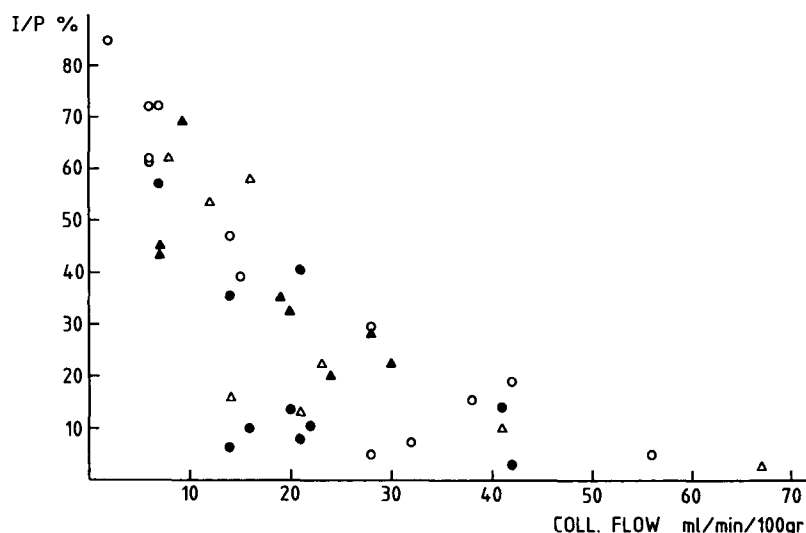


Table 2. Global and Regional Left Ventricular Contractility in 39 Dogs

	Superoxide Dismutase			Placebo		
	No.	LVEF (%)	RSS (%)	No.	LVEF (%)	RSS (%)
Control	18	58 ± 7	28 ± 6	21	61 ± 9	30 ± 10
Occlusion	18	36 ± 7	-6 ± 8	21	39 ± 12	-7 ± 13
Reperfusion						
4 h	8	30 ± 13	-10 ± 11	8	23 ± 14	-9 ± 9
48 h	10	40 ± 17	10 ± 14	13	45 ± 12	5 ± 14
1 wk	10	55 ± 7	19 ± 13	13	52 ± 12	8 ± 16

LVEF = global left ventricular ejection fraction; RSS = regional segmental shortening in the area of the left anterior descending coronary artery.

Global and regional (anterior wall) left ventricular contractility (Table 2). After 4 h of reperfusion, the decrease in global left ventricular ejection fraction and the outward systolic bulging of the postischemic area persisted in both the treated and placebo groups. Subsequently there was a gradual recovery of the ejection fraction in both groups. Regional wall motion in the left anterior descending artery area also recovered to a limited and variable degree.

Multiple regression analysis correcting for hemodynamic variables and wall motion in control conditions did not reveal any beneficial effect of superoxide dismutase treatment. The extent and the speed of recovery were not enhanced in treated dogs.

Myocardial high energy phosphate content (Table 3). Tissue ATP content, depressed after 4 h of reperfusion, was partially restored 1 week later, whereas tissue creatine phosphate content decreased during this time interval. After correcting for infarct size and high energy phosphate content in normally perfused myocardium, no effect of superoxide dismutase treatment could be demonstrated either after 4 h or 1 week of reperfusion.

Discussion

Experimental protocol. Assuming that irreversible reperfusion injury in the coronary occlusion/reperfusion setting exists, the discrepancies among various studies (5-16) examining the effect of treatment with free radical scavengers may in part be explained by differences in experimental design (1). In the present study, we tried to avoid some of the more obvious pitfalls. A relatively large number of dogs were randomized between placebo and active treatment and the investigators did not know the treatment allocation until completion of data analysis. The time frame using 90 min of

ischemia followed by reperfusion is well accepted in canine studies (6,7,10,14-16) and is not too distant from the clinical situation; the minimal duration of 4 h reperfusion brings the model within the reliable range for tetrazolium staining (18,26,27). Reperfusion by intravenous administration of a thrombolytic agent not only resembles the clinical situation, but also provides gradual reperfusion, preventing possible deleterious effects of abruptly removing a clamp (28,29).

We did not measure blood levels of superoxide dismutase and the drug was administered intravenously, in contrast to the intraatrial delivery route in some other studies; the timing (starting 30 min before reperfusion) and the dosage (a total of 2×10^6 IU over 60 min) of the intravenous superoxide dismutase administration, however, should have provided blood levels well within the acceptable range throughout the early phase of reperfusion.

We used the superoxide dismutase molecule in its non-conjugated form, which is known to have a short half-life of 10 to 20 min (1,12). Thus, protection from oxygen radicals may have been insufficient or absent during the later phases of reperfusion if at that stage oxygen radicals were generated (for example, by infiltrating neutrophils). Recent studies (10,16) have addressed this issue of duration of treatment by using superoxide dismutase conjugated to polyethylene glycol, thereby extending the half-life to 30 h; the results of these studies were discordant with respect to the effect of the drug on infarct size.

The use of iodinated radiopaque contrast medium in our experiments may be of some concern. Together with the thrombotic occlusion/thrombolysis feature of our protocol, the use of contrast angiograms made these experiments feasible in a closed chest preparation without any previous operation for instrumentation. Yet contrast agents have recently been shown (30-32) to affect granulocyte function.

Table 3. Myocardial High Energy Phosphate Content in Postischemic Tissue in 36 Dogs*

	Superoxide Dismutase			Placebo		
	No.	ATP	CrP	No.	ATP	CrP
4 h reperfusion	6	8.4 ± 6.0	33.5 ± 18.6	8	10.7 ± 6.0	36.9 ± 17.2
1 week reperfusion	9	17.6 ± 3.7	25.6 ± 6.4	13	12.2 ± 8.4	16.5 ± 12.2

*For technical reasons transmural biopsy samples from three dogs in the superoxide dismutase group were not available for analysis. Values are expressed in $\mu\text{mol/g}$ dry weight. ATP = adenosine triphosphate content; CrP = creatine phosphate content.

Inasmuch as neutrophils may contribute to lethal reperfusion injury and postischemic stunning of the myocardium, we cannot exclude the possibility that the use of contrast medium may have had an influence on our results. The role of neutrophils in the aforementioned phenomena, however, remains controversial (1).

Effect on infarct size. In assessing the effect of a drug on experimental infarct size in a canine model, the variables determining the infarct size in the control situation must explicitly be taken into account (23,33). This requirement is met by the statistical analysis of our data, which reveals the area at risk and the level of collateral flow to be the only predictors of infarct size, without any significant effect of superoxide dismutase treatment. With respect to collateral flow, it has been argued (5,7) that superoxide dismutase treatment may limit infarct size only in certain subgroups of dogs with collateral flow within a specific range. The available data, however, are contradictory because one study (7) found the greatest benefit in those dogs with the lowest collateral flow, whereas in the other (5), there was a beneficial effect only in dogs with a higher than average collateral flow.

One problem that this study shares with many previous investigations using the canine model is that of statistical power. These experiments are difficult, time-consuming and expensive and the number of animals must realistically be limited. Although it is difficult to associate exact power estimates with a multiple regression analysis, the statistical power is probably less than desirable, especially for smaller reductions in infarct size. Perhaps the time has come for someone to attempt a meta-analysis of all available data.

Postischemic stunning. Postischemic myocardial contractility could benefit from superoxide dismutase treatment by a more rapid recovery of contractile function because oxygen free radicals may play a role in the phenomenon of myocardial stunning (34). There was also the question whether a possible limitation of infarct size by superoxide dismutase would result in better preservation of left ventricular contractility in the long term. To examine these issues, left ventricular contractility was measured after different periods of reperfusion (4 h, 48 h and 1 week). Because acute ischemia, infarction and periinfarction stunning may affect global and regional contractility in different ways, measures of both global and regional wall motion were analyzed. The results expectedly showed a gradual recovery of left ventricular ejection fraction, which was not apparent until after 48 h. Regional wall motion in the postischemic area recovered with a similar temporal evolution, but to a variable degree, depending on the size of the infarction. These variables were not affected by superoxide dismutase treatment at any time during reperfusion. Given the lack of beneficial effect on infarct size, this outcome was to be expected after 1 week; the results after 4 and 48 h imply that superoxide dismutase does not alleviate the phenomenon of periinfarction myocardial stunning in this model.

These findings are in agreement with recent studies (15).

This inefficacy of superoxide dismutase after prolonged ischemia associated with subendocardial infarction is in contrast to the results (2-4) obtained after reversible ischemia, where free radical scavengers have generally been effective in alleviating postischemic stunning.

Biochemical recovery. We found no evidence of an effect of superoxide dismutase treatment on the recovery of the high energy phosphate content of postischemic myocardium. Individual values depended on the infarct size and we observed the expected increase in ATP from 4 h to 1 week of reperfusion. The decrease in creatine phosphate content from 4 h to 1 week can be ascribed to the overshooting of creatine phosphate repletion in viable tissue during the early reperfusion phase (35).

Conclusions. Superoxide dismutase treatment in this experimental model had no beneficial effect on infarct size or postischemic recovery of myocardial contractility and high energy phosphate content. This leaves us wondering about the missing link between the evidence for a role of oxygen radicals in reperfusion injury and the lack of therapeutic efficacy of free radical scavengers.

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